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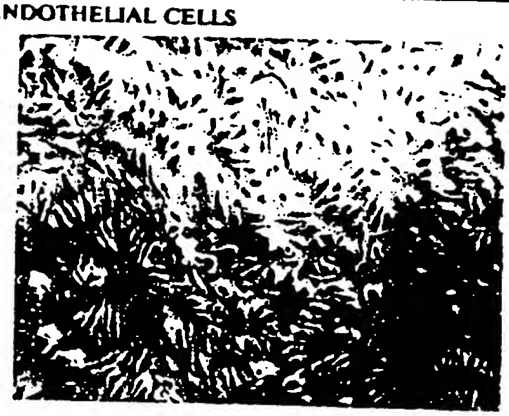

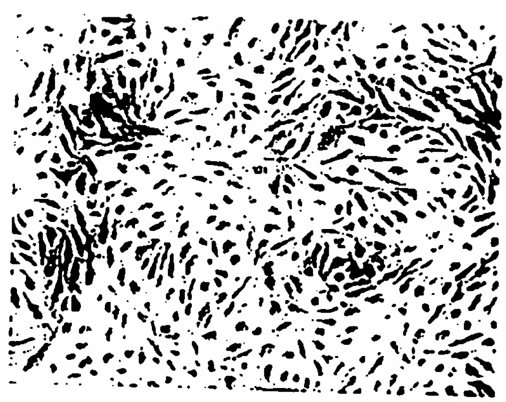

(57) Abstract

The present invention relates, in general, to endothelial cells. In particular, the present invention relates to a microvascular endothelial cell (or a cell line) obtained from human skin and immortalized and a method to establish such a line.

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IMMORTALIZATION OF ENDOTHELIAL CELLS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates, in general, to endothelial cells. In particular, the present invention relates to immortalized microvascular endothelial cells obtained from human skin.

Background Information

In the last 10 years, the accumulation of information about the endothelium has led to the realization that this is a tissue which is not just a target for injury, but by undergoing alterations in functions, metabolism and structure, it directly influences the evolution and outcome of vascular injury, inflammation and immune reactions like graft rejection and tumor metastasis (Cotran, R. (1987) Am. J. Pathol. 129, 3: 407-413). Factor VIII-related antigen is an endothelial cell product involved in the aggregation of platelets; megakaryocytes are the only other cell type known to express this antigen (Cotran, R. (1987) Am. J. Pathol. 129, 3: 407-413). Upon activation by molecules like Interleukin-1 and tumor necrosis factor, these cells up-regulate their expression of leukocyte specific adhesion molecules like ICAM-1 and ELAM-1 (Pober, J.S. (1988) Am. J. Pathol. 133, 3: 426-433; Butcher, E.C. et al. (1986) J. Cell. Biochem. 30, 2: 121-131); interferon-gamma is associated with the expression of Class II maj r histocompatibility antigens (Dvorak, H.F. et al.

(1986) Hum. Pathol. 17, 2: 122-127). Morphologically, endothelial cells can express facultative traits of tissues from which they were derived, including Weibel-Palade bodies and cobblestone growth pattern (Cotran, R. (1987) Am. J. Pathol. 129, 3: 407-413; Karasek, M.A. (1989) J. Invest. Dermatol. 93: 335-385).

Primary human microvascular endothelial cells have a limited life span of about 8-10 passages and have specific growth requirements. Early methods of tissue culture required the use of high concentrations of serum, Sarcoma 180 conditioned medium and multiple growth factors for optimal growth. Human serum requirements can be decreased or substituted with fetal bovine serum by incorporating 2% pre-partum maternal serum in the medium (Karasek, M.A. (1989) J. Invest. Dermatol. 93: 335-385). These cells can also be stimulated by the addition of agents such as cholera toxin, dibutyryl cAMP and isobutyl methyl xanthine which activate adenyl cyclase prolonging the growth rate and morphology (Tuder, R.M. et al. (1990) J. Cell Physiol. 142: 272-283). In the absence of cAMP, cultured vascular endothelial cells undergo pronounced changes in their morphology and functional properties; cells turn from epithelial to spindle shape and lose some of their ability to express HLA-DR antigens in response to interferon-gamma (Tuder, R.M. et al. (1990) J. Cell Physiol. 142: 272-283).

Various normal and neoplastic, as well as differentiated embryonic cells of human origin, have be n transformed and immortalized by intact SV40

virus including human umbilical cord endothelial cells (Sack, G.H., Jr. (1981) In Vitro 17, 1: 1-19; Gimbrone, M.A., Jr. et al. (1976) Cell 9: 685-693). Papova viruses constitute one of the simplest group of DNA tumor viruses and have been the most studied (Aaronson, S.A. (1970) J. Virol. 6, 4: 470-475). The SV40 transfected human endothelial cells did not exhibit Factor VIII related antigen expression nor show characteristic Weibel-Palade bodies (Gimbrone, M.A., Jr. et al. (1976) Cell 9: 685-693). Human umbilical vein endothelial cells have also been immortalized by exposure to murine sarcoma viruses containing the "v-ras" or "v-mos" oncogenes (Faller, D.V. et al. (1988) J. Cell Physiol. 134: 47-56). These cells expressed Factor VIII related antigen and contained Weibel-Palade bodies. An endothelial cell line derived from mouse lymph node stroma which retains most functional characteristics of normal mouse endothelial cells has been described (O'Connell, K.A., and Edidin, M. (1990) J. Immunol. 144, 2: 521-525); transient infection was performed using whole virus SV40 strain 4A to immortalize these cells.

The cells according to this invention exhibit a number of utilities. For example, the cells can be used to study the immediate adherence of HDMEC to graft vascular surfaces, for example: angioplasty and endarterectomy. The cells can also be used in pre-coating vascular grafts (with endothelial cells).

The cells can be used in metabolic studies of lipid and lipoprotein metabolism, arachidonic acid metabolism, hemostasis factors, and endothelial

derived vasoactive substances such as endothelin (ET). The cells can also be used in studies of angiogenesis, wound healing, leukocyte adherence and adhesion molecule expression (intracellular expression as well). Further, the cells can also be used in genetic studies aimed at the isolation of endothelial cell specific gene regulatory products and creation of cDNA libraries for endothelial cell specific genes.

One skilled in the art will appreciate that the cells of the invention can be used in pharmacologic studies as substrates for the screening of various agents as inhibitors of inflammation or modulators of cell adhesion molecule expression or in the cosmetic industry for toxicity testing. The cells, if tumorigenic, can be used in studies of endothelial cell tumor formation and potentially useful in specific problems, such as Kaposi sarcoma (or the transforming effects of chemicals or other agents on diploid human cells). The cells can also be used for viral or parasitic growth or detection. The cells can be used to produce products (for example: cytokines or lymphokines) which may be secreted into the medium or isolated from the cell surface.

SUMMARY OF THE INVENTION

It is a general object of this invention to provide a microvascular endothelial cell.

It is a specific object of this invention to provide a primate immortalized microvascular endothelial cell.

It is a further object of this invention to provide a primate immortalized microvascular endothelial cell line.

It is another object of this invention to provide a method of establishing a cell line of primate immortalized microvascular endothelial cells.

Further objects and advantages of the present invention will be clear from the description that follows.

In one embodiment, the present invention relates to a microvascular endothelial cell (or cell line) wherein the cell (or cell line) is obtained from a primate skin and is immortalized.

In another embodiment, the present invention relates to a method of establishing a cell line of immortalized microvascular endothelial cells derived from primate skin comprising:
(1) introducing DNA which encodes SV40 large T antigen into the cells and
(2) culturing the cell line.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. (A) Phase-contrast microscopy of a pure culture of human dermal microvascular endothelial cells (HDMEC) showing typical "cobblestone" morphology. (B) Phase-contrast microscopy of a culture of SV 40T-transformed human dermal microvascular endothelial cells. They also have a "cobblestone" appearance and are indistinguishable from non-transformed human dermal microvascular endothelial cells.

Figure 2. (A) Direct immunofluorescence microscopy of human dermal microvascular endothelial cells using acetylated LDL. The cells show granular cytoplasmic staining indicating uptake of the acetylated LDL, typical of endothelial cells. (B) Direct immunofluorescence microscopy of CDC/EU.HMEC-1. The staining is typical of endothelial cells and indistinguishable from that shown in Figure 2A.

Figure 3. (A) Phase-contrast microscopy of human dermal microvascular endothelial cells grown on matrigel. Long, tubular, cellular extensions are seen at eight hours of culture. This is typical of the morphologic differentiation that endothelial cells undergo when cultured on this matrix. (B) Phase-contrast microscopy of CDC/EU.HMEC-1 cells cultured on matrigel. Morphologic differentiation is noted that is very similar to that seen in Figure 3A.

Figure 4. CDC/EU.HMEC-1 (Transformed HDMEC) cells were grown in HDMEC medium supplemented with different concentrations of human serum. They were seeded at 1.7×10^4 cells/flask, cultured for eight days at 37°C, 5% CO_2, harvested, and counted. Cells grew at all concentrations of human serum that were tested but did so in a concentration-dependent fashion. (Untransformed human dermal microvascular endothelial cells will not grow below 20% normal human serum.)

Figure 5. CDC/EU.HMEC-1 or untransformed human dermal microvascular endothelial cells were seeded at 5×10^5 cells/25 cm^2 flask and grown for 10 days at 37°C 5% CO_2 with various concentrations of human serum. Cells were harvested every 24 hours

for 10 days and counted. (Open boxes = CDC/EU.HMEC-1, 30% serum; solid boxes = CDC/EU.HMEC-1, 1% serum; open diamonds = CDC/EU.HMEC-1, 0% serum; solid diamonds = HDMEC, 30% serum.) CDC/EU.HMEC-1 cells grow best with 30% human serum supplementation, but will survive at 0% human serum.

Figure 6. HLA-DR Expression on CDC-HMEC-1. Cells were untreated or treated with Interferon-gamma for seven days. Expression of HLA-DR was performed by FACScan analysis using PE-conjugated anti-human HLA-DR antiserum.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to human microvascular endothelial cells.

In one embodiment, the present invention relates to microvascular endothelial cells obtained from primate skin (preferably, human skin or foreskin) and immortalized. In a preferred embodiment, immortalization is effected by introducing DNA encoding SV-40 large T antigen into the skin-derived endothelial cells. A preferred cell line comprising such immortalized cell is designated CDC/EU.HMEC-1. This cell line has been deposited in accordance with the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA as ATCC Designation CRL 10636. The deposit was received January 8, 1991 and was accepted by this International Depository Authority.

The deposited human microvascular endoth lial cell line from human foreskin was immortalized by

transfecting freshly isolated cells with an eukaryotic vector containing the early region of the SV40 virus large T antigen. These cells (CDC/EU.HMEC-1) form microtubules on matrigel, they take up acetylated low density lipoprotein, express Factor VIII-related antigen and express HLA-DR antigen upon exposure to gamma-interferon; all of which are characteristics of normal microvascular endothelial cells. Additionally, these cells share the similar epithelioid cobblestone growth pattern of normal microvascular endothelial cells. Different from normal HDMEC, this new cell line requires low or no concentration(s) of human or fetal bovine serum for growth, has a shorter doubling time than HDMEC, do not require epidermal growth factor or hydrocortisone for growth and do not require gelatin or fibronectin-coated surfaces for attachment. Control cells which were not transfected and did not contain SV-40T DNA died off after two additional passages. Immortalized cells are thus defined as cells which remain alive after ten passages following introduction of DNA. Ideally, they replicate or divide indefinitely, maintain morphologic and physiologic characteristics of the tissue of origin, and grow at lower serum requirements and increased cell density. In particular, the DNA encodes the SV-40 large T antigen and can be introduced via transfection.

In a further embodiment, the present invention relates to a method of establishing a cell line of immortalized microvascular endothelial cells derived from primate skin (preferably human skin or foreskin). The method comprises introducing DNA

encoding the SV40 large T antigen into the cells under conditions such that the microvascular endothelial cells become immortalized and culturing the cell line.

The present invention is described in further detail in the following non-limiting examples.

EXAMPLES

The following protocols and experimental details are referenced in the examples that follow.

Isolation and culture of human dermal microvascular endothelial cells (HDMEC). HDMEC have been isolated from human foreskins by the following technique. Foreskins are cut into 3 mm squares and placed in phosphate-buffered saline (PBS) containing 0.3% trypsin (Sigma Chemical Co., St. Louis, MO) and 1% ethylenediamine tetracetic acid (Sigma) at 37°C for 10 minutes. The skin segments are washed with HBSS several times and placed in a petri dish in HBSS with the keratinized surfaced down. They are then individually compressed with the side of a scalpel blade to express microvascular fragments from the cut surfaces of the skin. The microvascular segments in 1 ml of HBSS are layered onto a 35% solution of Percoll (Pharmacia AB; Uppsala, Sweden) in HBSS that has been previously spun at 30,000 g for 10 minutes at 4°C to create a gradient. The gradient is then spun at 400 g for 15 minutes at room temperature. The fraction with a density less than 1.048 g per ml, which is rich in microvascular fragments, is removed. Those portions of the

gradient containing the microvascular segments are applied to a human fibronectin (Advanced Biotechnologies; Silver Spring, MD) precoated area 10 mm in diameter in the center of a 60-mm tissue-culture dish. The dishes are then incubated at 37°C in a moist incubator in 5% CO_2 overnight. Unattached cells are removed by washing with HBSS. The attached cells are then viewed with an inverted phase-contrast microscope in a biologic hood, and nonendothelial cells are removed by detaching them with a 25-gauge sterile needle. The growth medium for these cells consists of 200 ml Endothelial Basal Medium MCDB 131 (Clonetics, San Diego, CA), 75 ml human serum (Irvine Scientific, Santa Ana, CA), Dibutyryl CAMP 0.5 mM (Sigma Chemical Co., St. Louis, MO; Tuder et al. (1990) J. Cell. Physiol 142:272-283), Antibiotic-antimycotic solution 1% (final concentration) (Gibco, Grand Island, NY), hydrocortisone 2 µm (final concentration) and epidermal cell growth factor 5 ng/ml (final concentration). Transfected cells were grown in the same medium without CAMP. The resulting cell cultures are consistently 100% pure as assessed by morphological and immunochemical criteria. The normal cells can be continuously passaged up to 10 times.

Vector. The vector used in the transfection is designated as SV-40T. It has the sequence that codes for the transformation protein of SV-40 large T antigen and RSV-LTR cloned into the Eco R1 site of the PBR322 plasmid.

Transfection. Primary human foreskin endothelial cells were in their 6th passage from isolation when transfected. The procedure followed has been described by Graham and van der EB (Virology, 52:456-467, 1973). Some minor modifications were introduced to this procedure. All surfaces to which endothelial cells attach were pre-coated with a solution of 0.1% gelating in 0.01M phosphate buffer saline Ph 7.4 (PBS). The cells were plated onto 6-well plates at 3.5 x 10^5 cells/well and incubated @ 37°C and 5% CO_2 overnight before transfection. The amount of vector DNA used was 5 µg per well. Following the transfection procedure, the plates were incubated overnight @ 37°C with 5% CO_2, then the contents of each well transferred to individual 25 cm^2 plastic flasks with 0.2 µ filter cap (Costar). Some of the flasks contained dibutyryl cAMP 0.5mM (final concentration). Flasks were split 1:4 as they became confluent.

Detection of SV-40T viral antigen. To detect expression of the SV-40T in the immortalized endothelial cells (CDC/EU.HMEC-1) an enzyme-linked immunoassay was performed. Cell line SV-T2 a Balb/3T3 mouse embryo line infected with SV-40 (ATCC # CCL 163.1) was used as a positive control, a peripheral lymphocyte lysate was used as a negative control. Cell-free lysates were obtained by adding 0.5 ml of 0.5% deoxycholate.HCl in 0.01M PBS pH 8.2 and 100 µl of a 1 mM solution of phenylmethyl sulfuflouride in 95% ethanol to 10 million cells. This mixture was incubated @ 4°C for 30 minutes and then centrifuged @ 4000 RPM for 15 minut s @ 4°C.

The supernatant which possibly contained the SV-40 large T antigen was removed and saved.

For the ELISA test, the antigens were attached by suctioning onto nitrocellulose paper using a Minifold II Slot Blotter (Schleifher & Schuell, Keene, NH). After the attachment procedure, the sheets were incubated for 24 hr with 2% skim milk in 0.01 M PBS @ 4°C to prevent nonspecific binding. Afterwards, the skim milk solution is removed and the nitrocellulose washed three times with 0.1% Tween-20 in PBS @ RT. Mouse monoclonal antibody to the SV-40 large T viral antigen was added at a dilution of 1:1000 in PBS/Tween and incubated for 30 min @ 37°C on an orbital shaker. The monoclonal antibody to SV-40T antigen was obtained from the supernatant of the mouse hybridoma cell line Pab 101 (ATCC # TIB 117). The nitrocellulose sheets were then washed three times with Tween/PBS and an additional wash @ 37°C on the orbital shaker for 15 min. Goat anti-mouse horseradish peroxidase labelled monoclonal Ab (BioRad, Richmond, CA) was added at a 1:2000 dilution and allowed to incubate for 30 minutes @ 37°C shaking. Later, the nitrocellulose sheet was washed three times with Tween/PBS. A diaminobenzidine (Sigma Chemical Co., St. Louis, MO) (DAB) solution consisting of 25 mg of DAB, 50 ml of PBS, and 20 μl of 30% H_2O_2 was added and color allowed to develop. The nitrocellulose sheet was rinsed with deionized water to stop the enzymatic reaction.

Expression of SV-40T antigen. Cell-free lysates of CDC/EU.HMECI and SV-T2 (positive control) cells expressed the SV-40T viral antigen by ELISA and Western Blot. Negative control (human peripheral blood lymphocyte lysate) cells were negative.

Characterization of endothelial cell cultures. Representative cultures of HDMEC and SV 40T HDMEC (CDC/EU.HMEC-1) are characterized in three ways. Cultures are evaluated by inverted phase-contrast microscopy to determine whether the cells have the characteristic cobblestone morphology of endothelial cells. Cells are fixed in 90% methanol at -20°C for 10 minutes, washed and stained with a 1:40 dilution of rabbit anti-human factor VIII (Atlantic Antibodies; Scarborough, ME) for 30 minutes followed by FITC conjugated goat anti-rabbit IgG. They are washed three times and then viewed under a fluorescent microscope. Cells, unfixed, are incubated with acetylated low-density lipoprotein (10 μg/ml), labeled with 1,1'dioctadecyl-1,3,3,3'3'-tetramethyl indocarbocyanine perchlorate (Dil-AC-LDL) (Biomedical Technology, Inc.; Stoughton, MA) at 37°C in medium 199 without growth supplement or fetal calf serum for 4 hours. Dil-Ac-LDL is a biologic probe incorporated by living endothelial cells and, to a lesser extent, by monocytes or macrophages. The media is then removed, and the cells are washed twice and visualized in a fluorescence microscope with standard rhodamine excitation emission filters. Evaluation by all three techniques reveals that pure cultures of b th types of endothelial cells are routinely attained.

Differentiation of endothelial cells. Matrigel, an extract of the EHS sarcoma that contains basement membrane components (Collaborative Research; Bedford, MA), is applied to 24- or 96-well cell-culture plates as either a thick or thin film and then incubated at 37°C. This temperature induces gelling of the extract. HDMEC or CDC/EU.HMEC-1 are than plated onto the matrigel. The cells attach rapidly, and within 1-2 hours elongated processes are observed, and after 8 hours the endothelial cell cultures show abundant networks of branching and anastomosing cords of cells. By light microscopy, most of the cords show a central translucent structure along their long axis, which suggests the presence of a lumen. By 8 hours, the endothelial cells form an interconnected network of anastomosing cells that by low-power light microscopy have a "honeycomb" appearance. These endothelial cells express factor VIII-related antigen before, during, and after tube formation. They are also metabolically active, since they take up acetylated low-density lipoprotein. Transmission electron microscopy of cells cultured on matrigel for 18 hours demonstrates that cross-sections of the tube-like structures contain a lumen surrounded by cells. The membranes of the cells forming the lumen of the tubes connect with one another by interdigitating cytoplasmic processes.

Flow cytometry. Analysis of cell-surface m lecules on HDMEC and CDC/EU.HMEC-1 was performed using dir ct immunofluorescence and flow cytometry.

Cytometric analysis was performed on a FACscan Flow Cytometer (Becton-Dickinson, Inc.; Mountain View, CA). This instrument provides data regarding cell number, forward angle light scatter, side scatter, and red and green fluorescence. Approximately 10,000 cells per test sample were analyzed in these studies. HDMEC and CDC/EU.HMEC-1 to be analyzed in these studies will be removed from tissue-culture flasks with 6-10 ml of 5mM EDTA and 1% BSA to avoid any loss of trypsin or dispase-sensitive endothelial cell epitopes. After incubating for 30 minutes at 37°C, an equal volume of HBSS with CA++ and Mg++ is added to inactivate the EDTA, and the cells are washed three times. The cells are separated into aliquots of 10^6 cells/tube, pelleted, supernatant discarded, and 20 µl of undiluted monoclonal antibody is added. The cells are lightly vortexed and incubated for 30 minutes on ice. The cells are washed three times and then either stained with an appropriate second-step antibody or analyzed directly in one-step staining procedures.

EXAMPLE 1

Phenotypic Characterization of SV 40T HDMEC

The cell line, CDC/EU.HMEC-1 is in its 40th passage. Control cells which did not contain SV-40T DNA or cAMP growth supplement after transfection died off after two additional passages after transfection. CDC/EU.HMEC-1 assumed a "cobblestone" morphology when cultured on gelatin-coated tissue culture dishes when cultured in complete HDMEC media. Their morphology was essentially indistinguishable from HDMEC (Figur 1).

It was noted, however, that when allowed to become hyperconfluent, the CDC/EU.HMEC-1 were capable of growing to a higher density and that the cells appeared naturally smaller than HDMEC under these conditions.

CDC/EU.HMEC-1 and HDMEC both stained positively for Factor VIII when examined by direct immunofluorescence. Both types of cells also demonstrated uptake of acetylated low density lipoprotein after 4 hours exposure (Figure 2).

In order to determine whether CDC/EU.HMEC-1 were capable of morphologic differentiation into tubes, these cells were cultured on matrigel. It was previously shown that HDMEC will form capillary-like structures when cultured on the basement membrane-line matrix (Kubota, Y., et al. (1988) J. Cell Biol. 167:1589). CDC/EU.HMEC-1 demonstrated tube formation after 8 hours of culture on matrigel which was indistinguishable from that of HDMEC (Figure 3).

EXAMPLE 2

Proliferation Studies

It was previously demonstrated that optimal growth of HDMEC requires specialized growth medium supplemented with 30% human serum (Kubota, Y., et al. (1988) J. Cell Biol. 107:1589 and personal observation). Decreasing human serum to 20% or below essentially halts proliferation. In order to determine human serum requirements for CDC/EU.HMEC-1, comparison growth studies were performed with routine HDMEC medium supplemented with 30%, 20%, 10%, 5%, or 1% human serum. The results showed that although the cells grew best at

30% human serum, there was growth even at a concentration of 1% human serum (Figure 4). Population doubling time ranged from 53.6 hours for cells cultured in media containing 30% human serum to 85.6 hours for cells cultured in media containing 1% human serum. Further studies examining growth curves of CDC/EU.HMEC-1 showed that CDC/EU.HMEC-1 were capable of replicating in routine HDMEC culture media devoid of serum (Figure 5).

EXAMPLE 3

Cell Surface Molecule Expression

Previous studies have shown that HDMEC express a number of cell adhesion molecules on their surface including ICAM-1, LFA-3, CD44, and Class I, but lack constitutive expression of Class II molecules (Fleck, R. et al. (1986) J. Invest. Dermatol. 86:475). HDMEC and CDC/EU.HMEC-1 were compared for expression of these cell surface antigens. Both normal and transfected cells expressed ICAM-1, LFA-3, CD44, and Class I, but did not express Class II (Table 1). However, stimulation of HDMEC and CDC/EU.HMEC-1 with IFN-gamma (500 μ/ml, 72 hrs) resulted in induction of Class II cell surface antigen expression (Figure 6). The CDC/EU.HMEC-1 expressed more than 3 times as much CD44 and more than twice as much ICAM-1 and LFA-3 than did nontransfected HDMEC. Class I expression was roughly equivalent on both types of cells.

TABLE I. Comparison of expression of selected immunologically relevant cell-surface antigens on untransformed and CDC/EU.HMEC-

1 human dermal microvascular endothelial cells.

	HDMEC	CDC/EU.HMEC-1
ICAM-1	+	+
LFA-3	+	+
CD44	+	+
Class II	-	-
Class II*	+	+

*after stimulation for 72 h with 500 u/ml of IFN-gamma.

* * * * *

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

WHAT IS CLAIMED IS:

1. A microvascular endothelial cell wherein said cell is obtained from primate skin and is immortalized.

2. The microvascular endothelial cell according to claim 1 wherein said primate skin is human skin.

3. The microvascular endothelial cell according to claim 1 wherein said human skin is human foreskin.

4. The microvascular endothelial cell according to claim 1 wherein said cell contains DNA that encodes SV40 large T antigen.

5. The microvascular endothelial cell line CDC/EU.HMEC-1, ATCC # CRL 10636.

6. A method of establishing a cell line of immortalized microvascular endothelial cells derived from primate skin comprising:
(1) introducing DNA which encodes SV40 large T antigen into said cells under conditions such that said cells become immortalized and
(2) culturing said cell line.

7. The method of establishing a cell line according to claim 5 wherein said primate skin is human skin.

8. The method of establishing a cell line according to claim 6 wherein said human skin is human foreskin.

9. The method of establishing a cell line according to claim 5 wherein said introducing DNA is executed by transfection.

FIG. 1A

FIG. 1B

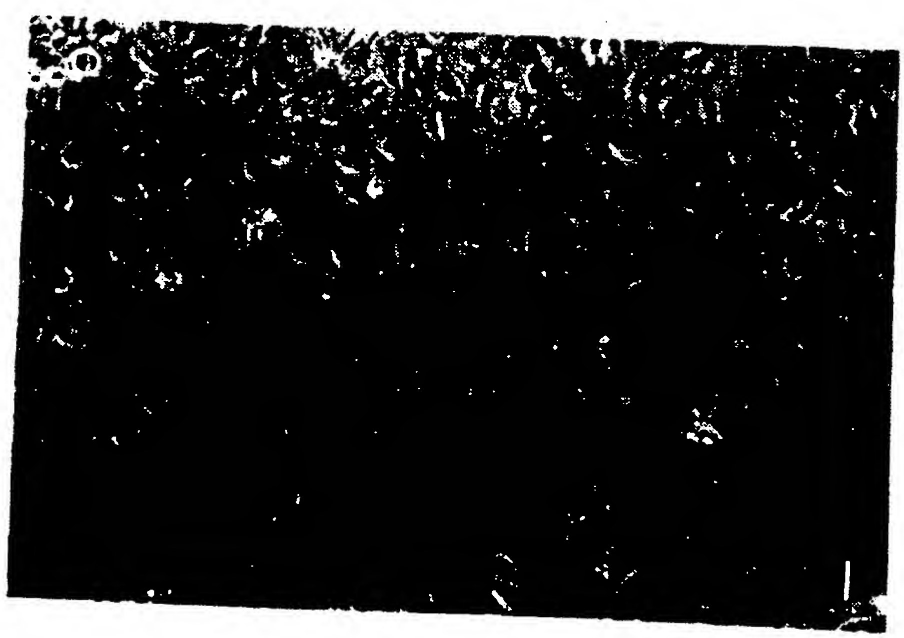


FIG. 2A

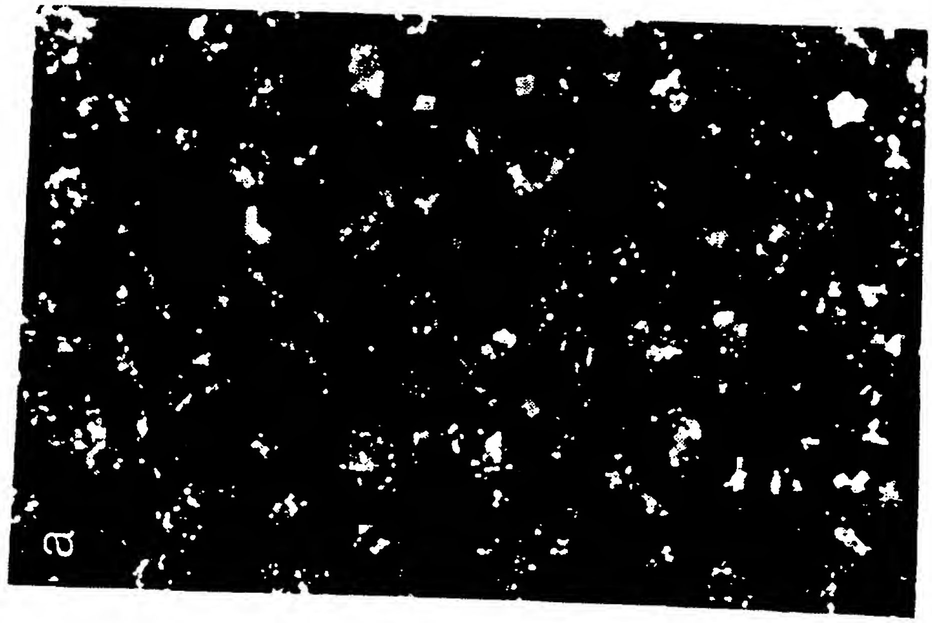


FIG. 2B

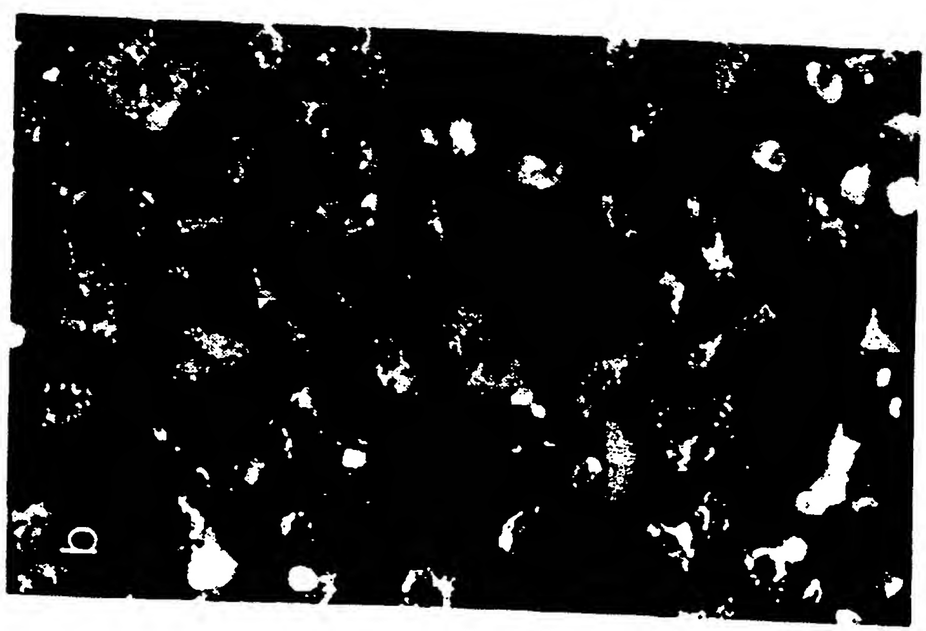


FIG. 3A

FIG. 3B

FIG. 4

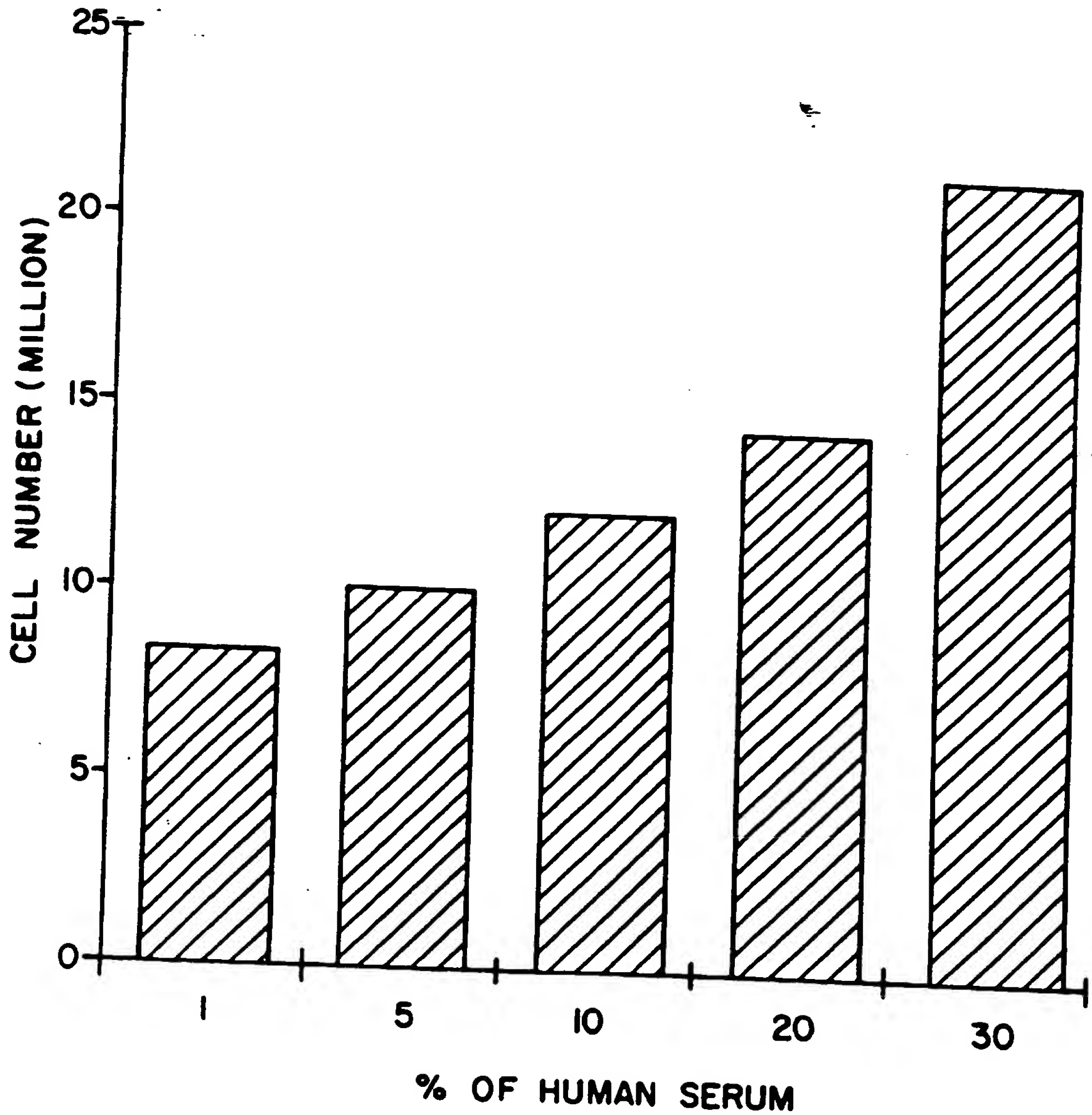
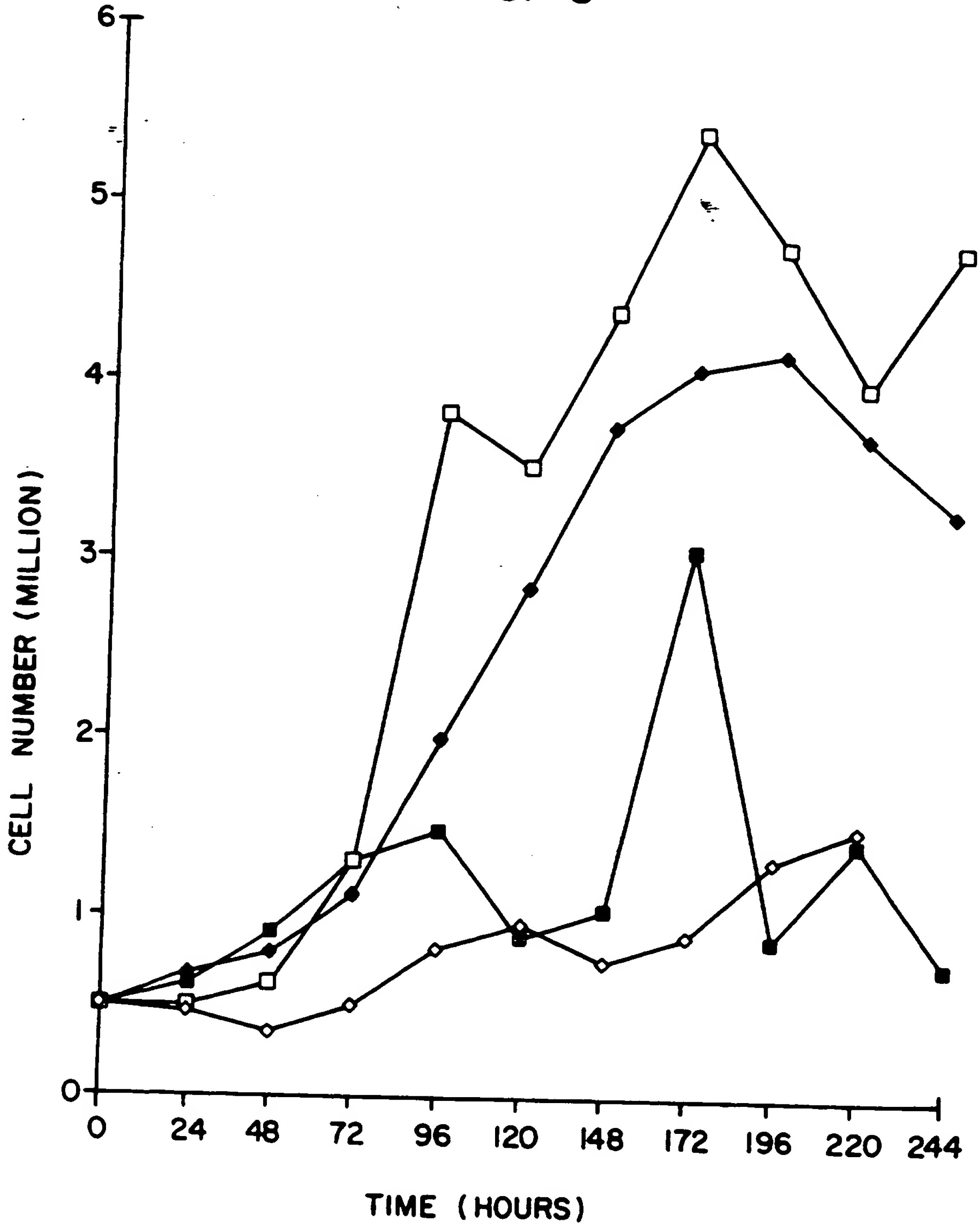


FIG. 5


6
5
4
3
2
1
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48
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96
120
148
172
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220
244
TIME (HOURS)

FIG. 6A

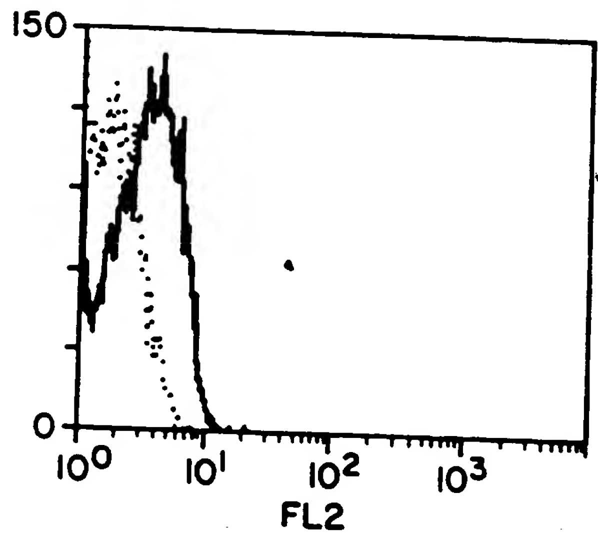
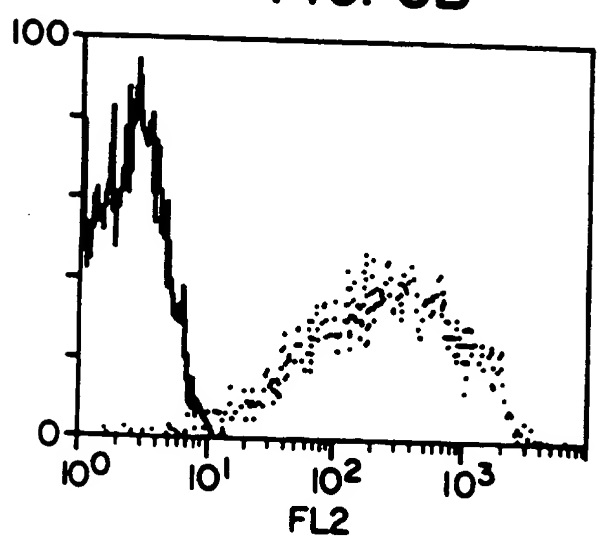


FIG. 6B



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02499

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12N 5/16, 5/22, 15/64
US CL : 435/240.2, 172.3

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/240.2, 172.3

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

CHEMICAL ABSTRACTS, APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	The Journal of Immunology, Volume 144, No. 2, issued 15 January 1990, K. A. O'Connell et al., "A MOUSE LYMPHOID ENDOTHELIAL CELL LINE IMMORTALIZED BY SIMIAN VIRUS 40 BINDS LYMPHOCYTES AND RETAINS FUNCTIONAL CHARACTERISTICS OF NORMAL ENDOTHELIAL CELLS", pages 521-525, see abstract.	1-9
Y	The FASEB Journal, Volume 4, issued October 1990, M. L. Brandi et al., "Establishment and characterization of a clonal line of parathyroid endothelial cells", pages 3152-3158, see entire document.	1-9
Y	JP, A, 0272866 (Kobayashi et al.) 13 March 1990, see entire document.	1-9
Y	Chemical Abstracts, Volume 113, issued 1990, T. Kobayashi et al., Preparation of human vascular endothelial cell line and its use for manufacturing tissue-type plasminogen activator", see page 568, column 2, Abstract No. 113896, JP, A, 0272866.	1-9

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATI N

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
17 Jun 1992	29 JUN 19..
International Searching Authority[1]	**Signature of Authorized Officer**[20]
ISA/US	JAMES KETTER

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	The Journal of Cell Biology, Volume 107, issued October 1988, Y. Kubota t al., "Role of Laminin and Basement Membrane in the Morphological Differentiation of human Endothelial Cells into Capillary-like Structures", pages 1589-1598, see entire document.	1-9

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

CORRECTED VERSION *

PCT

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 5/16, 5/22, 15/64 — A1

(11) International Publication Number: WO 92/17569

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(30) Priority data:
679,674 4 April 1991 (04.04.91) US

(71) Applicants: THE UNITED STATES OF AMERICA, as represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; National Institute of Health, Office of Technology Transfer, Box OTT, Bethesda, MD 20892 (US). EMORY UNIVERSITY [US/US]; 1380 South Oxford Road, N.E., Atlanta, GA 30322 (US).

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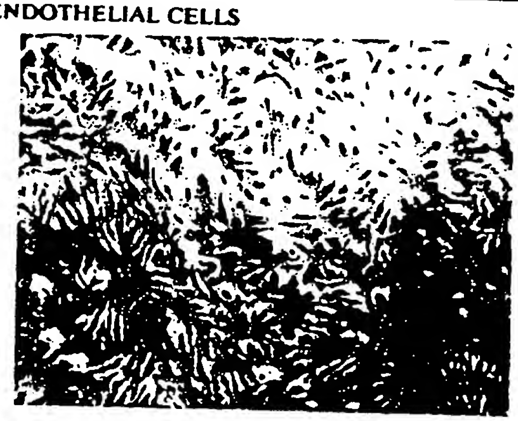
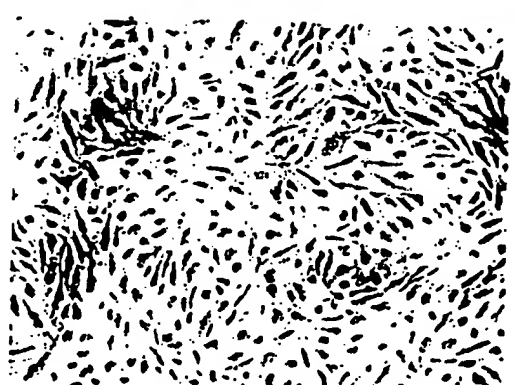
(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: IMMORTALIZATION OF ENDOTHELIAL CELLS





(57) Abstract

The present invention relates, in general, to endothelial cells. In particular, the present invention relates to a microvascular endothelial cell (or a cell line) obtained from human skin and immortalized and a method to establish such a line.

* (Referred to in PCT Gazette No. 29/1992, Section II)

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